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Ink and Vinegar, a Simple Staining Technique for Arbuscular-Mycorrhizal Fungi

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We developed a reliable, inexpensive, and simple method for staining arbuscular-mycorrhizal fungal colonizations in root tissues. Apart from applications in research, this nontoxic, high-quality staining method also could be of great utility in teaching exercises. After adequate clearing with KOH, an ink-vinegar solution successfully stained all fungal structures, rendering them clearly visible.

Healthy, fertile soils are characterized by the presence of a diverse population of microorganisms, an important component of which are arbuscular-mycorrhizal (AM) fungi (12). The arbuscular mycorrhiza is a symbiotic association formed between the roots of members of over 80% of all families of land plants and a small group of common soil-borne zygomycetic fungi (*Glomales*). In general, this association is beneficial for both partners. The host plant receives mineral nutrients from outside the root's depletion zone via the extraradical fungal mycelium, while the heterotrophic mycobiont obtains photosynthetically produced carbon compounds from the host (18). Research into the establishment and role of mycorrhizal associations in natural ecosystems is of fundamental importance. Although data are available from several North American and European ecosystems, few data have been obtained from developing countries where the maintenance of AM fungal populations could be essential for sustainable agriculture. The availability and cost of chemicals used for staining of AM fungi within root tissues, a basic technique in AM research, can be constraints in some countries.

Phillips and Hayman (15) developed a method of staining AM fungal structures in roots that uses trypan blue. Trypan blue is listed by the International Agency for Research on Cancer as a possible carcinogen (9). Another frequently applied technique (3) uses the possibly carcinogenic dye chlorazol black E (10). Acid fuchsin, which also is used to stain AM fungi in roots (7), is also a suspected carcinogen (5). In addition, HCl, although used at a low concentration, is frequently applied for the acidification of roots after clearing with KOH (7, 15).

The use of such chemicals should be reduced for health and safety reasons. Contact with caustic chemicals may cause skin irritation (2), and their vapors may irritate the eyes, nose, throat, and lungs (16, 17). For environmental reasons it is preferable, wherever possible, to find substitutes for harmful chemicals. The "International Directory of Mycorrhizologists" lists more than 1,000 mycorrhizologists in 77 countries worldwide (6); thus, we estimate that tens of thousands of root samples are stained per year. In an attempt to eliminate some of the hazardous compounds, a modified procedure for stain-

TABLE 1. Comparison of different inks for staining of AM fungi in roots

Color	Company	Staining result	Comments
Purple	Waterman	Fungus not stained	Not suitable
Green	Reynolds	Fungus not stained	Not suitable
Blue	Shaeffer	Fungus not stained	Not suitable
	Kreuzer	Fungus partially stained	Not suitable
	Pelikan	Fungus stained; good contrast	Suitable
Red	Lamy	Fungus not stained	Not suitable
	Pelikan	Fungus not stained	Not suitable
	Parker	Fungus stained; roots after destaining light red; good contrast	Suitable; especially interesting in combination with staining for viable AM mycelium (13)
Black	Reform	Fungus not stained	Not suitable
	Carrefour	Fungus stained; roots after destaining light blue; good contrast	Suitable
	Pelikan	Fungus stained; roots after destaining light grey; good contrast	Suitable
	Shaeffer	Fungus stained; roots after destaining light brownish; excellent contrast	Suitable
	Cross	Fungus stained; roots after destaining light blue; good contrast	Suitable

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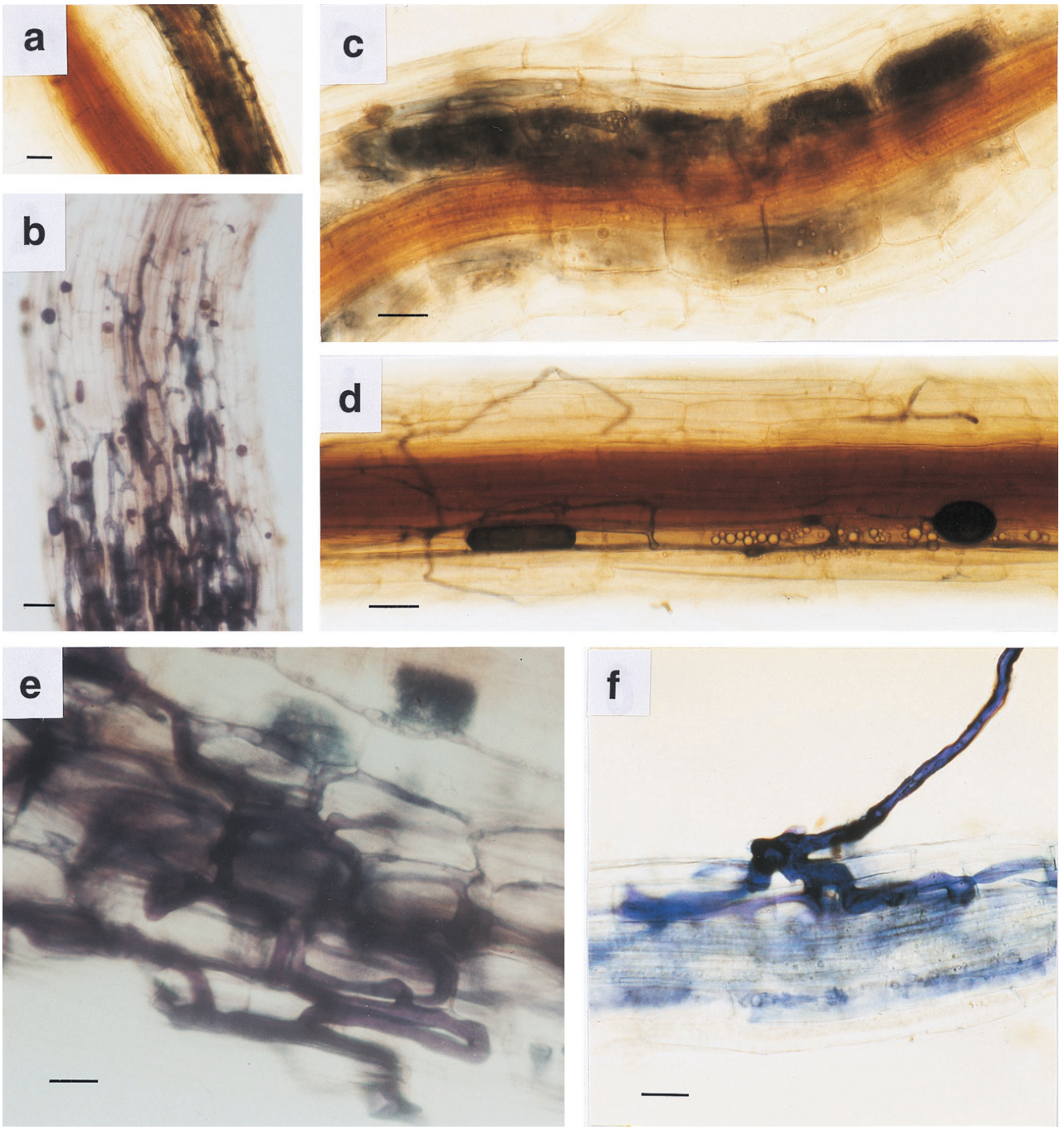

FIG. 1. *G. mosseae*-colonized roots stained with an ink-vinegar solution. Roots in panels a to e were stained with black ink (Shaeffer). (a) Colonized (dark) and noncolonized ryegrass roots. The colonized root is easily distinguishable from the noncolonized root. Bar = 25 μm. (b) Individual hyphae in both heavily and partially colonized sections of a bean root are clearly visible. Bar = 20 μm. (c) Arbuscules in ryegrass root tissue. Bar = 20 μm. (d) Vesicles and internal hyphae in ryegrass root tissue. Bar = 20 μm. (e) Arbuscules and internal hyphae in bean root tissue. Bar = 5 μm. (f) Penetration unit of *G. mosseae* in a ryegrass root stained with blue ink (Pelikan). Bar = 20 μm.

ing of AM fungi in roots has been proposed (11); however, the carcinogenic dye trypan blue is still used. Recently, a simple staining technique with an ink–25% acetic acid solution for screening of *Pseudocercosporella herpotrichoides* infection in wheat leaves was developed (14). Our objective was to determine whether this technique can be adapted for staining of AM fungi in roots, thus replacing toxic chemicals with nontoxic but equally effective products.

Biological materials. Seeds of plants from different families having differing root characteristics (bean [*Phaseolus vulgaris* L.], soybean [*Glycine max* L.], cucumber [*Cucumis sativus* L.], maize [*Zea mays* L.], wheat [*Tritium aestivum* L.], barley [*Hordeum vulgare* L.], and ryegrass [*Lolium perenne* L.]) were surface sterilized by soaking in 0.75% sodium hypochlorite for 5 min, rinsed with tap water, and germinated in vermiculite. Seedlings (7 days old) were transferred to a steam-sterilized

TABLE 2. Staining of colonized roots with trypan blue (standard method) or with black ink (Shaeffer)

Plant	% of roots stained with[a]:	
	Trypan blue	Ink
Wheat	43 ± 3	45 ± 5
Bean	60 ± 6	58 ± 3
Cucumber	35 ± 4	37 ± 6

[a] Means (± standard errors) of root samples from five replicate plants per species and treatment.

(40 min, 120°C) mixture of usual silicate sand (article number 142, 265; Bourbeau et Fils Inc., Charlesbourg, Québec, Canada), TurFace (baked clay substrate mechanically broken into pieces with diameters of approximately 5 mm) (Applied Industrial Materials, Corp., Buffalo Grove, Ill.), and soil (2:2:1, vol/vol/vol) (from the tree nursery of the Université Laval, Québec, Canada) containing an inoculum of one of the three AM fungi (*Glomus mosseae* [BEG 12; La Banque Européenne des Glomales, International Institute of Biotechnology, Kent, England], *Glomus intraradices* [DAOM 197198; Biosystemic Research Centre, Ottawa, Canada], or *Gigaspora margarita* [DAOM 194757]). Plants grown in a growth chamber (day/night cycle of 16 h, 22°C/8 h, 20°C; relative humidity, 50%) were harvested 7 weeks after inoculation. After removal of the growth medium, roots were rinsed with tap water.

For each of the plant species, roots from at least five replicate plants per treatment were stained. When inks successfully stained the fungi in the roots, root samples from at least another 20 replicates were performed.

For comparisons of results obtained with an ink to those by a standard method using a dye, trypan blue (15), 7-day-old cucumber, bean, and wheat seedlings were inoculated with *G. mosseae*. Four weeks after inoculation, plants were harvested and their roots were stained. The percentage of root colonization was determined by the line-intersect method (1), with the colonization in root systems of five plants per treatment quantified.

Staining. Roots were cleared by boiling in 10% (wt/vol) KOH (15) (boiling times differed according to the type of plant [see Table 3]) and then rinsed several times with tap water. Cleared roots were boiled for 3 min in a 5% ink-vinegar solution with pure white household vinegar (5% acetic acid). Roots were destained by rinsing in tap water (acidified with a few drops of vinegar) or by rinsing in pure vinegar (destaining times differed according to the type of ink [see Table 3]). The following inks were tested: purple (Waterman, Paris, France), green (Reynolds, Valence, France), red (Parker, Boston, Mass.; Lamy, Heidelberg, Germany; and Pelikan, Hannover, Germany), blue (Pelikan; Kreuzer, Hannover, Germany; and Shaeffer, Ft. Madison, Iowa), and black (Pelikan; Reform, Heidelberg, Germany; Carrefour, Paris, France; Shaeffer; and Cross, Lincoln, R.I.).

Black ink (Shaeffer) was also tested with a 10% ink solution in 25% acetic acid, as described by Mauler-Machnik and Nass (14). After being destained, roots were kept in tap water at room temperature in the laboratory.

Clearing of bean, soybean, and maize roots for 5 min and of cucumber, wheat, barley, and ryegrass roots for 3 min in 10% KOH provided transparent roots suitable for staining. The green, the purple, two each of the blue and red, and one of the black inks did not stain the fungal tissue. However, one blue, one red and the other black inks all stained intraradical and extraradical AM fungal structures (Table 1; Fig. 1), allowing observation of these structures within the roots. Colonized roots were easily distinguished from noncolonized roots (Fig. 1a). Individual hyphae in heavily and partially colonized sections of roots were clearly visible (Fig. 1b). Arbuscules, vesicles, intraradical and extraradical hyphae, and penetration units were all stained by the inks (Fig. 1c to f). For photographic and assessment purposes, the best contrast was achieved with Shaeffer black ink. After destaining, roots were pale brownish red, whereas fungal structures remained black (Table 1; Fig. 1a to e). Staining with the 10% black ink–25% acetic acid solution, as proposed by Mauler-Machnik and Nass (14), gave no better results than staining with a 5% black ink-vinegar (= 5% acetic acid) solution. No differences in the degree of root colonization were observed when roots of plants of different plant families were stained with trypan blue (standard method [15]) or an ink (Table 2).

Destaining of the blue and the black inks was most successful when tap water (with a few drops of vinegar) rather than vinegar was used (Table 3).

Staining of all three AM fungi—*G. mosseae*, *G. intraradices*, and *G. margarita*—by the black ink-vinegar solutions gave excellent results. After storage for 6 weeks in tap water at room temperature in the laboratory, fungal structures were as clearly visible as immediately after completion of the staining process.

Red ink also stained AM fungi (Table 1). To obtain the best results, bean roots had to be boiled in KOH for 15 min and ryegrass roots had to be boiled for 5 min (Table 3). When roots were boiled in KOH for shorter periods of time, the plant tissue remained heavily stained even after destaining and the fungal structures could not be differentiated. Destaining in vinegar gave better contrast than destaining in tap water (Table 3).

Our results with ink-vinegar solutions show that after an adequate clearing time, good staining and destaining results are obtained by the method presented. The time for clearing of root tissues must be adjusted depending upon the plant species studied. This method gives not only excellent results but also safe alternatives to the hazardous, toxic, and potentially carcinogenic chemicals used in usual staining techniques, as vine-

TABLE 3. Recommended clearing, staining, and destaining procedures for different ink colors

Ink color	Procedure[a]		
	Clearing	Staining (min)	Destaining
Black or blue	Time depends on type of root (e.g., bean, 5 min; ryegrass and cucumber, 3 min); then, rinse several times with tap water	3	Rinse for a minimum of 20 min with tap water acidified with a few drops of vinegar
Red	Time depends on type of root (e.g., bean, 15 min; ryegrass and cucumber, 5 min); then, rinse several times with tap water	3	Rinse for 10 min with vinegar

[a] General procedures: clearing, boil in 10% KOH; staining, boil in 5% ink-vinegar.

gar, which is used in human nutrition, is obviously not harmful and ink, because it is used by children, is subject to strict regulations and must be nontoxic in every respect (4).

As not all inks tested were capable of staining AM fungal tissue, and as it might not always be possible to obtain inks from the companies we used in our study, a preassay should be performed with each specific ink considered.

Grace and Stribley (8) and Koske and Gemma (11) proposed modification of the staining method for AM fungi developed by Phillips and Hayman (15) which eliminate as many toxic compounds as possible. Our method, with ink-vinegar solutions, reduces still further the list of potentially hazardous chemicals needed. Moreover, by our method, the acidification procedure that follows the clearing of roots with KOH (15) can be omitted.

Our method provides a simple and safe technique with easily obtainable compounds and also may be applicable for staining of other root-colonizing fungi. For example, on roots of wheat plants inoculated with *Rhizoctonia cerealis*, extensive mycelium was clearly visible with some hyphae penetrating the root (results not shown). This inexpensive staining technique might stimulate AM research in parts of the world where financial resources for scientific studies are highly limited. Moreover, the nontoxic chemicals used make it an excellent technique for teaching situations.

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